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Review article

Genetically engineered human neural stem cells for brain repair in neurological diseases

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Abstract

Neural stem cells (NSCs)of the central nervous system (CNS) have recently received a great deal of attention and interest for their therapeutic potential for neurological disorders. NSCs are defined as CNS progenitor cells that have the capacity for self-renewal and multipotent potential to become neurons or glial cells. Recent studies have shown that NSCs isolated from mammalian CNS including human can be propagated *in vitro* and then implanted into the brain of animal models of human neurological disorders. Recently, we have generated clonally derived immortalized human NSC cell lines via a retroviral vector encoded with v-*myc* oncogene. One of the human NSC lines, HB1.F3, was utilized in stem-cell based therapy in animal models of human neurological disorders. When F3 human NSCs were implanted into the brain of murine models of lysosomal storage diseases, stroke, Parkinson disease, Huntington disease or stroke, implanted F3 NSCs were found to migrate to the lesion sites, differentiate into neurons and glial cells, and restore functional deficits found in these neurological disorders. In animal models of brain tumors, F3 NSCs could deliver a bioactive therapeutically relevant molecules to effect a significant anti-tumor response intracranial tumor mass. Since these genetically engineered human NSCs are immortalized and continuously multiplying, there would be limitless supply of human neurons for treatment for patients suffering from neurological disorders including stroke, Parkinson disease, Huntington disease, ALS, multiple sclerosis and spinal cord injury. The promising field of stem cell research as it applies to regenerative medicine is still in infancy, but its potential appears limitless, and we are blessed to be involved in this exciting realm of research.

Keywords: Human neural stem cell; F3 human NSCs; Cell therapy; Lysosomal storage disease; Stroke; Parkinson disease; Huntington disease; Brain tumor

Contents

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1. Introduction

Stem cells are multipotent cells that are both self-renewing and under the right conditions or right signals can give rise to many different cell types that make up the organism. Stem cells hold great therapeutic potentials for injury and disease in human. Two types of mammalian pluripotent stem cells, embryonic stem (ES) cells derived from the inner cell mass of blastocysts and embryonic germ (EG) cells obtained from post-implantation embryos, have been identified and these stem cells give rise to various organs and tissues [1–4]. In addition to ES cells and EG cells, tissue specific stem cells could be isolated from various tissues of more advanced developmental stages such as bone marrow mesenchymal stem cells, hematopoietic stem cells and neural stem cells among others. Existence of multipotent neural stem cells (NSCs) has been known in developing or adult rodent central nervous system (CNS) tissues [5–9]. These cells have the capacity to grow indefinitely and multipotent potential to differentiate into three major cell types of CNS, neurons, astrocytes and oligodendrocytes. In human, existence of NSCs with multipotent differentiation capability has also been reported in embryonic and adult human brain [10–16]. Among these studies, a study has demonstrated that in a group of cancer patients who had bromodeoxyuridine (BrdU) infusion for diagnostic purposes and later died, BrdU-labelled proliferating cells that co-labeled with neuronal marker were found in granular layer of hippocampal dentate gyrus [17]. It is evident from this study that new neurons are continuously being generated in adult human CNS. Why, in neurodegenerative diseases or after CNS injury, then the NSCs are ineffective in brain repair? Three different considerations may underlie the inability of adult NSCs to perform effective repair: (a) Although NSCs are present, their number is not enough to perform brain repair; (b) NSCs are unable to reach to the pathology site; (c) Microenvironment in the site of pathology is not favorable for the NSCs to expand and conduct repair. These difficulties in brain repair might be overcome if sufficient number of NSCs as well as simultaneous trophic factor environment is delivered in the injury site. Our recent transplantation studies using conditionally immortalized human neural stem cell line in several neurological disease models indicate that this approach is feasible.

2. Clonally isolated stable immortalized human neural stem cell lines

Recently continuously dividing immortalized cell lines of NSCs have been generated by introduction of oncogenes and these immortalized NSC lines have advantageous characteristics for basic studies on neural development and cell replacement therapy or gene therapy studies: (a) NSC cell line can be expanded to large numbers in culture in short time (24–36 h doubling time); (b) NSC cells are homogeneous since they were generated from a single clone; (c) stable expression of therapeutic genes can be achieved readily [8,18–20]. Immortalized NSCs have emerged as highly effective source for genetic manipulation and gene transfer into the CNS *ex vivo*; immortalized NSCs were genetically manipulated *in vitro*, survive, integrate into host tissues and differentiate into both neurons and glial cells after transplantation to the intact or damaged brain [8,13,19,21]. Primary cultures of fetal human telencephalon cells (at 15 weeks gestation) were infected with a retroviral vector carrying v-myc oncogene and clones with continuously dividing NSCs selected. Both *in vivo* and *in vitro* these cells were able to differentiate into neurons and glial cells and populate the developing or degenerating CNS [13]. More recently we have generated new lines of immortalized human NSCs using a retroviral vector carrying v-myc. HB1.F3, one of the newly generated human NSCs, is a clonally isolated, multipotent human neural stem cell line, and has the ability to self-renew, differentiate into cells of neuronal and glial lineages in both *in vivo* and *in vitro* [22,23]. HB1.F3 human neural stem cell line expresses nestin and Musashi-1, both cell type-specific markers for NSC and shows normal human karyotype of 46,XX. Following transplantation into the brain of animal models of focal ischemia [24,25], intracerebral hemorrhage [26], Huntington disease [27,28], Parkinson disease [29] and lysosomal storage disease MPS VII [30], F3 human NSCs successfully integrated into host brain parenchyma and provided functional recovery in these experimental animals.

3. Cell fate determination in neural stem cells

Existence of multipotent neural stem cells (NSCs) has been known in developing or adult mammalian central nervous system (CNS) tissues including human [5–9]. These cells have the capacity to grow indefinitely and pluripotent potential to differentiate into three major cell types of CNS, neurons, astrocytes and oligodendrocytes. However, differentiation of NSCs into neurons and glial cells occurs spontaneously when they are grown on top of permissive substrates. It is therefore desirable to obtain a large number of selected subpopulation of neurons or glial cells from continuously growing human NSCs by controlling the differentiation steps more rigorously. One way to accomplish this goal is transfer of relevant signal molecules or regulatory genes into the NSCs [31]. Differentiation of NSCs in neurosphere into neurons could be increased by treatment with NT3, NT4 and platelet derived growth factor

(PDGF) [32]. There was a large increase in TH-positive neurons when neurospheres derived from fetal rat midbrain were grown in the presence of bFGF and astrocyte-conditioned medium [33]. Another approach to induce specific subpopulation of neurons or glial cells is to introduce neurogenesis associated genes into NSCs and induce cellular differentiation. Since NSCs are readily transfectable with DNAs and stable sublines expressing transgenes could be generated, NSCs could be transfected with a neurogenic transcription factor gene and consequently induced to differentiate into a specific neuronal cell type. It has been known previously that Notch signaling, a member of basic helix loop helix (bHLH) transcription factors, is an important pathway that controls a broad spectrum of cell fate and shown to induce glial cells in the CNS. Transient activation of Notch1 in rat NSC cell line induced commitment of these cells to astrocytes [34]. A recent study from my laboratory has demonstrated that following transfection of HB1.F3 human NSCs with a full length coding region of NeuroD, a neurogenic bHLH transcription factor, F3.NeuroD cells differentiated into neurons expressing neuron-specific markers neurofilament proteins and tubulin βIII, two types of K+ current and tetrodotoxin-sensitive Na+ current [22]. For the induction of oligodendrocytes from NSCs, recent studies have identified Olig1 and Olig2, novel bHLH transcription factors, as important intracellular signal to regulate the differentiation processes of oligodendrocytes [35,36]. We have recently transfected HB1.F3 human NSC cells transiently with Olig2 DNA and obtained an induction of oligodendrocytes as shown by expression of oligodendrocyte specific markers galactocerebroside and O4 antigens in these cells (Kim, unpublished data). It is well known that both extrinsic and heritable intrinsic signals play important roles in generating cellular diversity in the CNS. It is now possible to obtain a large number of selected populations of neurons or glial cells from continuously growing human NSCs by introducing relevant signal molecules or regulatory genes into the NSC cell line.

4. Neural stem cell-based therapy for lysosomal storage diseases

Cell replacement and gene transfer to the diseased or injured CNS have provided the basis for the development of potentially powerful new therapeutic strategies for a broad spectrum of human neurological diseases including stroke, neurodegenerative diseases such as Parkinson disease, Huntington disease, amyotrophic lateral sclerosis and Alzheimer disease, and also for lysosomal storage diseases in babies.

Lysosomal storage diseases affect 1 in 7700 birth and most of affected babies show a diffuse CNS involvement [37]. Currently no effective treatment is available for most of the lysosomal diseases, since the blood–brain-barrier (BBB) bars entry of enzyme preparations into the brain [38]. In animal models of lysosomal diseases, therapeutic levels of enzymes could be achieved in the brain by direct inoculation of genetically engineered mouse neural progenitor cells [39], fibroblasts [40] and amniotic epithelial cells [41].

In mouse model of mucopolysaccharidosis type VII (MPS VII), a lysosomal storage disease caused by a genetic defect in the activity of β-glucuronidase, in collaboration with Jikei research group headed by Dr. Y. Eto, we have genetically modified the HB1.F3 immortalized human NSC line to overexpress β-glucuronidase, and transplanted the cells into the cerebral ventricles of MPS VII mouse brain [30]. Transplanted human NSCs migrated extensively in the brain, produced high levels of β-glucuronidase enzyme, reduced enzyme substrate to the normal levels, and cleared lysosomal storage in the neuronal cytoplasm [30]. The results indicate that human NSCs would serve as an excellent gene transfer vehicle for the treatment of diffuse CNS pathology in human lysosomal storage diseases.

5. Neural stem cell-based therapy for stroke

Stroke represents the second highest among the cause of death in Japan, Korea and China. There are two major types of stroke and they are ischemia and intracerebral hemorrhage. Ischemic stroke caused by abrupt and near-total interruption of cerebral blood flow, produces ischemic changes in the striatum and cortex, leading to a long-term sensorimotor deficit. Once damage from a stroke occurred, little can be done to restore premorbid functions, and although numerous neuroprotective agents have been clinically tried, no specific agents replaced the lost neurons, improved the deteriorated functions, and reduced the long-term sequelae [42]. There have previously been several reports of cell transplantation in the brain of ischemia animal models. Various cellular sources such as rodent mesenchymal stem cells [43,44], mouse hippocampal cells [45], immortalized mouse neural precursor cells [46–48], human umbilical cord blood cells [49], and human teratocarcinoma-derived neurons [50,51] were grafted into the ischemic brain, and reduced the neurological deficits induced by experimental brain ischemia. A previous study has reported that in human patients with ischemic infarct, some functional improvement was found with implantation of human teratocarcinoma-derived neurons [52]. More recently, a report has indicated that intravenous infusion of patients' own bone marrow-derived stromal cells induced a slight improvement in clinical outcome [53].

Recently, we have successfully introduced immortalized human NSCs (HB1.F3.LacZ) intravenously in rat

models of ischemia in which medial cerebral artery is occluded for 60 min and demonstrated that the human NSCs selectively migrated into lesioned brain sites, differentiated into new neurons and glial cells, and improved the functional deficits in these animals [24,25]. NSCs can circumvent blood–brain barrier and migrate to the specific pathologic areas of brain with trophism. We introduced HB1.F3 immortalized human NSCs intravenously via tail veins and F3 NSCs migrated into the the adult rat brain with transient focal cerebral ischemia or with cerebral hemorrhage. Transplanted human NSCs migrated to the lesion side, differentiated into neurons and astrocytes, and a large number of the grafted human NSCs survived in the lesion sites for up to 12 weeks. Three-12 weeks post-transplantation, functional improvement was observed in the transplanted animals compared with non-grafted controls on rotarod and turning-in-an-alley tests. Our results demonstrate that intravenously transplanted human NSCs differentiate into various neural cell types and compensate for the lost functions in rats caused by the focal cerebral ischemia [25] and intracerebral hemorrhage [26].

ICH is a lethal stroke type, as mortality approaches 50% and severe neurological disability in survivors is common. Since medical therapy against ICH such as mechanical removal of hematoma, prevention of edema formation by drugs, and reduction of intracranial pressure, shows only limited effectiveness, alternative approach is required [54,55]. Previous studies have indicated that NSCs or neural progenitor cells engrafted in animal models of stroke survive and ameliorate neurological deficits in the animals [56,57], raising the possibility of therapeutic potential of NSCs for repair of damaged brain in ICH models. Experimental ICH was induced by intrastriatal administration of bacterial collagenase in adult mice. One week after the surgery, the rats received human NSCs labeled with β-gal intravenously. Intravenously transplanted human NSCs selectively migrated into lesioned brain sites, differentiated into new neurons and glial cells, and improved the functional deficits in these animals [26]. More recently human NSCs were transplanted intracerebrally in the vicinity of the ICH lesion sites, and extensive migration of transplanted NSCs was identified in the peri-hematomal areas and differentiated into neurons (β-gal/MAP2+ and β-gal/NF+) or astrocytes (β-gal/GFAP+). The NSC-transplanted group showed better functional performance on rotarod test and limb placing after 2–8 weeks as compared with the control animals (Lee et al., unpublished results).

6. Cell therapy for Parkinson disease

Parkinson disease (PD) is an attractive target for these therapeutic approaches because the degeneration and cell death of dopaminergic neurons in substantia nigra, is well characterized. Previous studies have reported that implantation of fetal ventral mesencephalic cells into the caudate and putamen of PD patients provided a marked improvement in clinical course in these patients [58–60]. However, this therapeutic approach has faced with grave limitations because of ethical, religious and logistical problems in acquiring fetal tissues. To circumvent these difficulties, it is necessary to procure alternative source of cells producing dopamine or L-dihydroxyphenylalanine (L-DOPA), a dopamine precursor. Successful application of *in vivo* gene transfer to the CNS will depend on the identification of suitable cells that can serve as carriers for a wide range of potentially therapeutic transgenes and provide platforms for functionally efficient expression and secretion of transgene products. Immortalized CNS-derived neural stem cells (NSCs) have recently been introduced as potentially interesting candidates for this purpose. NSC transplantation has the potential to prevent or restore anatomic of functional deficits associated with injury or disease in the CNS via cell replacement, the release of specific neurotransmitters, and/or the production of factors that promote neuronal growth and regeneration.

Earlier studies have focused on the development of genetically engineered cell lines that overexpress tyrosine hydroxylase (TH), the rate-limiting step in catecholamine biosynthesis [61,62] or neurotrophic factors that promote survival of dopaminergic cells [63,64]. However, parkinsonian animal models grafted with genetically modified TH cells have shown only partial restoration of behavioral and biochemical deficits. The failure of this approach may be related to the limited availability of cofactor, tetrahydrobiopterin (BH4), because neither the dopamine-depleted striatum nor cells used for gene transfer possess a sufficient amount of BH4 to support TH activity [65]. Therefore, it is necessary to transfer GTP cyclohydrolase 1 (GTPCH1) gene that is the first and rate-limiting enzyme in the BH4 biosynthetic pathway [65]. Recently in my laboratory, we have modified HB1.F3 human NSCs to produce L-DOPA by double transduction with cDNAs for human TH and rat GTPCH1. Transplantation of these cells in the brain of PD rat model led to enhanced L-DOPA production *in vivo* and induced long-term functional recovery following intrastriatal transplantation [29,66].

It appears that differentiation of NSCs into neurons and glial cells occurs spontaneously without any apparent regulatory factor(s) controlling it when NSCs were plated onto substrates. It would be advantageous if one could control the differentiation steps more rigorously so that one can obtain a large number of desired neuronal or glial subpopulations from continuously growing population of human NSCs. One way to

accomplish this goal is transfer of relevant signal molecules or regulatory genes into the NSC cell line. Recently subline of F3 human NSC cells expressing the dopaminergic neuron-associated nuclear receptor Nurr-1 was generated [67]. The cell line did not produce dopamine or L-DOPA when they were grown in normal serum-containing medium, but they became dopaminergic when culture medium was supplemented with FGF-8, sonic hedgehog protein and astrocyte-conditioned medium as previously reported. It appears that F3.Nurr-1 human NSCs are capable of becoming dopaminergic neurons when given appropriate extracellular signals.

7. Proactive stem cell therapy in Huntington disease

Huntington disease (HD) is an autosomal dominant neurodegenerative disorder characterized by involuntary choreiformic movements, cognitive impairment, and emotional disturbances [68,69]. Despite identification of the HD gene and associated protein, the mechanisms involved in the pathogenesis of HD remain largely unknown and thus hamper effective therapeutic interventions. Transplantation of fetal human brain tissue may serve as a useful strategy in reducing neuronal damage in HD brain and a recent study has documented improvements in motor and cognition performance in HD patients following fetal cell transplantation [70]. This trial follows previous reports that positive effects of fetal striatal cell transplantation to ameliorate neuronal dysfunction in animal models of HD [71] and that striatal graft tissue could integrate and survive within the progressively degenerated striatum in transgenic HD mouse model [72]. The latter study is consistent with results obtained from HD patients indicating survival and differentiation of implanted human fetal tissue in the affected regions [73]. A major limiting factor in the transplantation of cells is the difficulty in supplying sufficient amounts of embryonic striatal tissue and the concomitant ethical issues associated with the use of human embryonic tissue. An ideal source of cell transplantation in HD would be neural stem cells (NSCs) which could participate in normal CNS development and differentiate into regionally appropriate cell types in response to environmental factors. In this regard previous studies have shown that NSCs isolated from embryonic or adult mammalian CNS can be propagated *in vitro* and subsequently implanted into the brain of animal models of human neurological disorders including HD [74]. Transplantation of NSCs to replace degenerated neurons or genetically modified NSCs producing neurotrophic factors have been used to protect striatal neurons against excitotoxic insults [8]. At present, little is known regarding whether implantation of NSCs prior to neuropathological damage could alter the progressive degeneration of striatal neurons and motor deficits that occur in HD. This question is important since genetic study of Huntington disease gene mutation [75] and neuroimaging can provide details on factors involved in the progression of HD [76,77] suggesting early intervention using brain transplantation could be effective in "would-be" HD patients.

Systemic administration of 3-nitropropionic acid (3-NP) in rodents leads to metabolic impairment and gradual neurodegeneration of the basal ganglia with behavioral deficits similar to those associated with HD [78,79]. Recent studies have demonstrated that brain-derived neurotrophic factor (BDNF) could block neuronal injury under pathological conditions in animal models of HD [80,81].

We have investigated the effectiveness of transplantation of human NSCs in adult rat striatum prior to striatal damage induced by the mitochondrial toxin 3-NP [27]. Systemic 3-NP administration caused widespread neuropathological deficits similar to ones found in HD including impairment in motor function and extensive degeneration of cresyl violet+ neurons, glutamic acid decarboxylase (GAD)+ neurons and calbindin+ striatal neurons. Animals receiving intrastriatal implantation of human NSCs one week prior to 3-NP treatments exhibited significantly improved motor performance and increased resistance to striatal neuron damage compared with control sham injections. In contrast, transplantation of human NSCs at 12 h following 3-NP administration did not show any protective effects against 3-NP-induced behavioral impairment and striatal neuronal damage. The neuroprotection provided by the proactive transplantation of human NSCs in the rat model of HD appears to be contributed by brain-derived neurotrophic factor (BDNF) secreted by the transplanted human NSCs. Active production of BDNF by human NSCs *in vivo* and *in vitro* was firmly established by studies using RT-PCR, immunocytochemistry, dot-blot, and ELISA. These novel findings suggest that proactively transplanted human NSCs were well integrated in the striatum and supported the survival of host striatal neurons against neuronal injury induced by neurotoxin 3-NP [27].

More recently, we have intravenously transplanted HB1.F3 human NSC cells 1 week after the induction of striatal pathology in rat using quinolinic acid (QA), an excitotoxic amino acid, and found that the NSC grafts survived well in QA animals and induced a behavioral improvement in apomorphin-induced turning response [28].

8. Neural stem cells for brain tumor therapy

Malignant brain tumors such as glioblastoma multiforme remain virtually untreatable and lethal despite

extensive surgical excision and radiotherapy and chemotherapy [82]. Their resistance to treatment is associated with their exceptional migratory nature and ability to insinuate themselves seamlessly and extensively into normal brain tissues, often migrating great distances from the primary tumor masses. These cells are responsible for the recurrent tumor growth near the borders of the resectution cavity. NSCs by virtue of their inherent migratory and tumor-tropic properties, represent a novel and potentially powerful approach for the treatment of invasive tumors. Utilized as a delivery vehicle to target and disseminate therapeutic gene products throughout tumor sites, NSCs may overcome major obstacles facing current gene therapy strategies by infiltrating tumor mass selectively and aggressively. A previous study has shown that when murine NSC cell line C17.2 cells carrying oncolysis-promoting pro-drug activating enzyme cytosine deaminase (CD) were grafted into glioma-bearing animals and prodrug 5-fluorocytosine was injected systematically, there was an 80% reduction in resultant tumor mass [83]. Recently our collaborators at the Harvard have demonstrated that human NSCs carrying PEX gene, "surround" the invading glioblastoma tumor cells, "chasing down" infiltrating tumor cells, and "attack and kill" tumor cells, causing a 90% reduction in tumor volume [84]. PEX is a naturally occurring fragment of human metalloproteinase-2, act as an inhibitor of glioma proliferation, migration and angiogenesis [85]. When human NSC line HB1.F3 carrying CD enzyme gene was transplanted intracranially at distant sites from the tumor, the donor NSCs migrate through normal tissue and selectively "home in" to the glioblastoma tumor mass and upon administration of prodrug 5-fluorocytosine, 80–85% reduction in tumor volume was demonstrated [86].

Despite multimodality treatments, medulloblastomas are incurable in more than 30% of patients. In addition, the current therapies lead to long-term disabilities. Based on the extensive tropism of NSCs for malignant gliomas demonstrated in previous studies, we studies the potential of hNSCs to deliver a therapeutic gene, using HB1.F3 cells engineered to secrete the prodrug activating enzyme cytosine deaminase (HB1.F3.CD) was examined. For the *in vivo* therapeutic proof of principal, animals bearing intracranial medulloblastomas (human Daoy cell line) were inoculated ipsilaterally with HB1.F3.CD cells followed by systemic 5-fluorocytocine treatment. Histological analyses showed that NSCs migrate to the tumor boundary leading to a 76% reduction of tumor volume in the treatment group. We demonstrate for the first time the potential of human NSCs as an effective delivery system to target and disseminate therapeutic agents to medulloblastomas (Kim et al., unpublished).

These results suggest the effective use of inherently migratory NSCs as a delivery vehicle for targeting therapeutic genes and vectors to refractory, migratory and invasive brain tumors such as glioblastoma and medulloblastoma.

9. Conclusions

There are a number of issues to be clarified before adoption of NSCs for stem-cell based therapy is widely accepted in clinical medicine such as which type of stem cells are most suitable for cell replacement or gene therapy in patients with neurological disorders or brain injury. Since neurons could be derived not only from NSCs, but also from embryonic stem (ES) cells, embryonic germinal (EG) cells, bone marrow mesenchymal stem cells, umbilical cord blood hematopoietic stem cells and even from fat cells or skin cells, the most pressing question is which cells are best suited for cell replacement therapy. Since the presence of NSCs in adult CNS is known, it is only a matter of time before neurons and glial cells are isolated from adult CNS tissue samples. There are ongoing debates as to why embryonic or fetal materials should be used to generate stem cells when stem cells could be isolated from adult tissues. However, most of the research up to now indicates that embryonic or fetal stem cells are significantly more versatile and plastic than adult counterparts. For this reason, use of embryonic or fetal tissues will continue so as the religious and ethical debates

Opposing use of such tissue samples continuously dividing immortalized cell lines of human NSCs as generated by introduction of oncogenes have advantageous features for cell replacement therapy and gene therapy and the features include that human NSCs are homogeneous since they were generated from a single clone, can be expanded to large numbers *in vitro*, and stable expression of therapeutic genes can be achieved readily. Immortalized human NSCs have emerged as highly effective source of cells for genetic manipulation and gene transfer into the CNS *ex vivo* and once transplanted into damaged brain they survive well, integrate into host tissues and differentiate into both neurons and glial cells.

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